Progress in Molecular Imaging in Endoscopy and Endomicroscopy for Cancer Imaging

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ABSTRACT
Imaging is an essential tool for effective cancer management. Endoscopes are important medical instruments for performing *in vivo* imaging in hollow organs. Early detection of cancer can be achieved with surveillance using endoscopy, and has been shown to reduce mortality and to improve outcomes. Recently, great advancements have been made in endoscopic instruments, including new developments in optical designs, light sources, optical fibers, miniature scanners, and multimodal systems, allowing for improved resolution, greater tissue penetration, and multispectral imaging. In addition, progress has been made in the development of highly-specific optical probes, allowing for improved specificity for molecular targets. Integration of these new endoscopic instruments with molecular probes provides a unique opportunity for significantly improving patient outcomes and has potential to further improve early detection, image guided therapy, targeted therapy, and personalized medicine. This work summarizes current and evolving endoscopic technologies, and provides an overview of various promising optical molecular probes.

Keywords: endoscopy, endomicroscopy, optical imaging, molecular probes

1. INTRODUCTION
Molecular imaging is an important emerging technology for improving clinical management of cancer and for advancing laboratory-based methodologies to study cancer biology. Molecular imaging can play an important role in clinical care of cancer by improving our ability to perform risk stratification, screening, surveillance, guiding biopsy, staging, prognosis, planning and guidance of therapy, monitoring therapy efficacy, and assessing recurrence. With rapid technological advances, the future role

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for molecular imaging may also include pre-symptomatic detection, targeted therapy, and personalized medicine [1].

Conventional imaging modalities such as magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), computed tomography (CT), and ultrasound (US) have distinct advantages and limitations. MRI detects the relaxation times of magnetic dipoles, such as hydrogen atoms in water and organic compounds, after a radiofrequency pulse, and then generates MR signals. MRI offers spatial resolution on the millimeter scale with simultaneous physiological and anatomical correlation. However, MRI requires long scan and post processing times and has relatively low sensitivity, thus requiring high doses (typically micrograms to milligrams) of magnetic contrast agents. PET and SPECT record high- and low-energy γ– rays, respectively, emitted from within the body. These imaging modalities have very high sensitivity but relatively low spatial resolution. CT images are acquired based on the extent of X-ray absorbance by tissue. CT provides high-resolution anatomical images especially for bones and tumors, but has poor soft tissue resolution and limited molecular imaging applications. US has high temporal resolution, can achieve imaging in real time, and is inexpensive and safe. However, the use of US in molecular imaging has so far been limited to intravascular targets. Thus, these conventional imaging modalities have relatively low spatial resolution in comparison to optics and some risk due to radiation. Furthermore, most of these techniques require a significant cost for initial start-up, maintenance, and infrastructure development, limiting access to patients in less developed countries.

Optical imaging is an emerging technology that offers several unique advantages and is being combined with molecular probes to improve specificity. Optics is non-ionizing, and can provide resolution on the micron scale, allowing for sub-cellular visualization [2]. Light over a broad range of colors allows for multispectral techniques to visualize multiple molecular targets simultaneously. Therefore, heterogeneous gene expression patterns found in tumors can be better characterized. Multispectral imaging requires the use of several molecular probes that have different emission wavelengths but similar binding kinetics, and can achieve greater detection sensitivity as a panel. Another advantage of optical imaging is the ability to collect images in real time in comparison to other imaging modalities, such as MRI and PET.

Endoscopes are thin, flexible instruments that provide a macroscopic view of the large mucosal surfaces in hollow organs internal to the human body. Miniature optics with large divergence angles is used that can generate a large field-of-view (FOV) with high spatial resolution. Endomicroscopy employs high numerical aperture (NA) optics to provide a small FOV with micron-level resolution for observing sub-cellular features. It requires scaling down the size of a conventional microscope design into a miniature package [3]. These instruments provide a unique opportunity for early cancer detection and prevention by allowing therapy (biopsy or resection) to be performed concurrently with diagnosis.

Several endoscopic imaging methods have been developed for imaging mucosa over a large field-of-view (FOV). White light endoscopy (WLE) uses visible reflectance from the mucosal surface, and is limited to visualizing flat and depressed lesions that

have minimal morphological changes [4]. Narrow band imaging (NBI) uses optical filters to reduce (narrow) the spectral bandwidth of the illumination in the green regime to enhance absorption from hemoglobin to improve visualization of vascular patterns and fine mucosal surface textures, but the results for early detection of cancer vary. Autofluorescence imaging (AFI) uses blue excitation (390–470 nm) to detect fluorescence from endogenous fluorophores, such as collagen, flavins, and porphyrins; however, clinical studies have demonstrated lack of specificity [5, 6].

Novel optical designs and scanning mechanisms are being developed to improve imaging performance for both endoscopy and endomicroscopy. This paper highlights some of these emerging technologies, focusing on recent advances in instrumentation as well as optical molecular probes. The aim of this review is to outline promising endoscopic instruments, molecular probe platforms, and integration of these technologies for cancer imaging. Section 2 summarizes existing endoscopic technologies, categorized as macroscopic and microscopic approaches, including pre-clinical and clinical results for each technology. The following sections address molecular probes for optical imaging and recent endoscopic molecular imaging.

2. ENDOSCOPIC INSTRUMENTS FOR MOLECULAR IMAGING

2.1. Endoscope (Macroscopic Imaging) - Scanning Fiber Endoscope

The scanning fiber endoscope (SFE) scans in a spiral pattern by a tubular piezoelectric actuator to create an image with a large FOV. Red, green, and blue (RGB) laser light (440, 532, and 635 nm wavelengths) is delivered through the scanning fiber and focused onto the mucosal surface with a multi-lens assembly located in the distal tip. The reflectance and fluorescence light is collected by a ring of multimode optical fibers arranged around the perimeter of the instrument. The SFE imaging technology was first developed for early detection of cancer in the esophagus, pancreatic duct, and peripheral airways using reflected white light [2, 7].

Recently, the SFE technology has been adapted for collection of fluorescence to perform molecular imaging. In a recent study, SFE demonstrated potential for use in molecular imaging [8]. The detection system of the multispectral endoscope (Figure 1A) uses longpass (wavelength $\lambda_{LP} = 450$ nm) and notch ($\lambda_{N1} = 532$ nm and $\lambda_{N2} = 632.8$ nm) filters to reject the reflectance component (RGB laser excitation) of the light from the collection fibers. The distal tip of multispectral SFE with 1.6 mm outer diameter and 10 mm rigid distal tip is shown in Figure 1B. To prove the multispectral and molecular imaging concept, three different peptides that bind specifically to colonic dysplasia were labeled with three different fluorescent dyes that have excitation wavelengths matching the three laser excitation wavelengths. Diethylaminocoumarin-3-carboxylic acid (DEAC) with absorption and emission peaks at 432 and 472 nm, 5-carboxytetramethylrhodamine (TAMRA) with absorption and emission peaks at 541 and 568 nm, and CF633 with absorption and emission peaks at 630 and 650 nm were used. Images of KCCFPAQ-DEAC (blue), AKPGYLS-TAMRA (green/yellow), LTTHYKL-CF633 (red), and unlabeled peptide droplets collected with a multispectral SFE are shown in Figure 1C-D. This study demonstrated the capability of the multispectral SFE to simultaneously detect multiple labeled peptides over the visible spectrum.

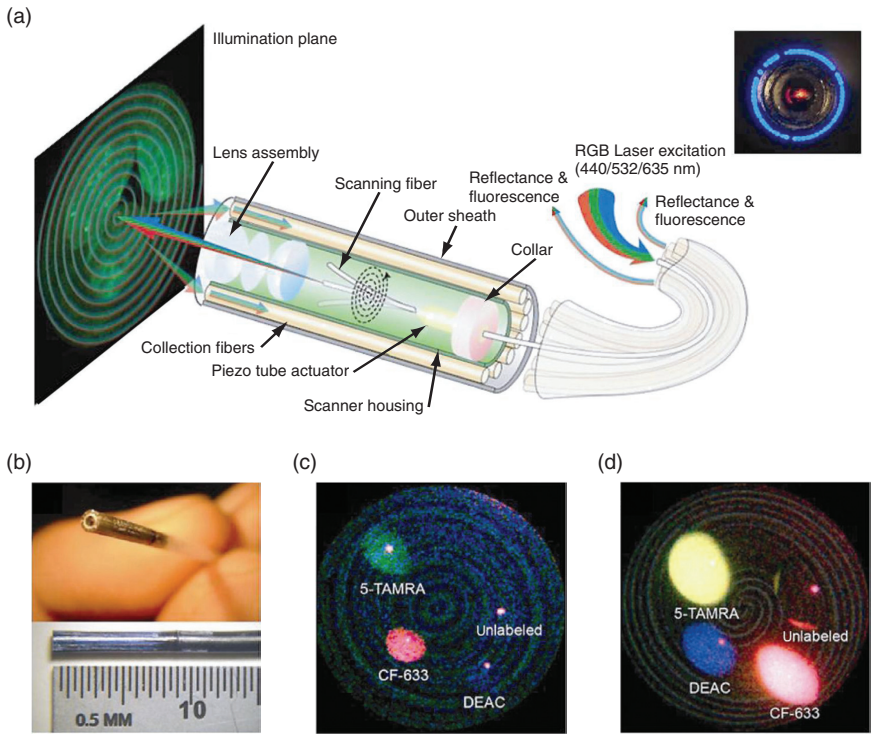


Figure 1. (A) Multispectral scanning fiber endoscope (SFE). Inset shows illumination fiber (red) and a ring of multimode optical fibers for light collection (blue). (B) *En face* (top) and side-view (bottom) of distal tip of multispectral SFE. (C, D) Images of labeled and unlabeled peptide droplets at concentrations of 1 μM (C) and 100 μM (D). Used with permission [8].

2.2. Endomicroscope (Microscopic Imaging)

2.2.1. Confocal Endomicroscope

A confocal endomicroscope performs high resolution optical sectioning over a small FOV, similar to a laboratory microscope, but in a package that is scaled down in size to millimeter dimensions. A single mode optical fiber placed on the main optical axis between the objective lens and the detector acts as a "pinhole" to allow only the light from a small focal volume below the tissue surface to be collected. A high NA objective lens is used to illuminate and collect light to achieve subcellular resolution with maximum light collection. For endoscope compatibility, the diameter of the overall package must be reduced to ~5 mm or less. As a result, the working distance (WD), FOV, and tissue penetration depth are usually reduced. Confocal endomicroscopes can be used to guide biopsies, and have been demonstrated in a number of clinical studies to detect cancer in the digestive tract, bladder, cervix, ovary, oral cavity, and lungs, with some recent findings from human clinical trials summarized below.

2.2.1.1. Single Axis Architecture

Two confocal endomicroscopes that use the single axis architecture have been commercialized and employed in the clinics. In the first approach, a fiber scanning design (Optiscan Pty Ltd, Victoria, Australia) is integrated into the insertion tube of a medical endoscope (EC-3870K, Pentax Precision Instruments, Tokyo, Japan). A semiconductor laser provides excitation at 488 nm wavelength. The distal tip of the fiber is laterally scanned by a tuning fork mechanism, and axial scanning is performed by a shape memory alloy actuator that moves the focal volume over a distance of up to 250 μm below the tissue surface. This system uses an objective that has an NA ~0.6, yielding a transverse and axial resolution of 0.7 and 7 μm, respectively.

Kiesslich *et al*. demonstrated this endomicroscope in the colon in 69 patients. After intravenous injection of fluorescein sodium, neoplastic changes were identified with a sensitivity of 97.4% and specificity of 99.4% [9]. In another study of 9 gastric cancer patients, neoplastic changes could be identified with a sensitivity of 92.6% and 88.8%, specificity of 100% and 100%, and accuracy of 96.3% and 94.4% on evaluation by an endoscopist and a pathologist, respectively [10]. However, comparing confocal endomicroscopy with other techniques such as NBI and chromoendoscopy, the advantage of confocal imaging in terms of accuracy in identifying colorectal polyps was found to vary from study to study [11, 12].

Another approach is based on a coherent fiber bundle. A miniprobe (Mauna Kea Technologies, Paris, France) passes through the standard instrument channel of medical endoscopes. Excitation is also provided at 488 nm as in the first approach, and scanning is performed at the proximal end of the fiber bundle in the instrument control unit using a set of oscillating and galvo mirrors. In this design, axial scanning is not available, and thus optical sections at different depths are achieved by using separate miniprobes that have different working distances. An objective with NA of ~0.6 is employed to provide a transverse and axial resolution of 2.5–5 μm and 15–20 μm, respectively.

This instrument is much smaller in diameter, and was demonstrated in the biliary ducts in 37 patients who underwent endoscopic retrograde cholangiopancreatography (ERCP) for bile duct stone removal or bile duct stenosis. Malignant strictures were clearly differentiated from normal common bile duct walls using the CholangioFlex probe. Neoplasia was predicted with 83% sensitivity and 75% specificity [13]. In a similar study, the Cellvizio CholangioFlex probe was used in a 102-patient study to develop and validate a standard descriptive classification for images collected in the pancreaticobiliary system [14]. A consensus definition of the specific criteria of biliary and pancreatic imaging findings for indeterminate strictures was developed.

Probe-based confocal endomicroscopy has also been employed for molecular imaging. A heptapeptide, that contains a VRPMPLQ sequence, was identified using phage display technology [15]. This peptide was labeled with fluorescein, applied topically to the colonic mucosa of patients undergoing routine colonoscopy, and showed specific binding to sporadic human colorectal adenomas. The peptide bound to dysplastic colonocytes with 81% sensitivity and 82% specificity.

2.2.1.2. Dual-Axes Architecture

In the dual-axes design, two optical fibers and two low-NA objectives are employed to separate the illuminating and collecting light paths, resulting in a sub-cellular resolution in three dimensions (3D), longer WD, and deeper tissue penetration [16]. The longer WD also allows for post-objective scanning, resulting in scalability and a larger FOV. Scanning is performed with a tiny micro-mirror fabricated using Micro Electro Mechanical Systems (MEMS) mirror technology. This instrument can be scaled down to millimeter dimensions.

Piyawattanametha *et al*. developed an MEMS-based dual-axes confocal endomicroscope with a 5.5-mm outer diameter for clinical imaging in the gastrointestinal tract [17]. Figure 2A shows miniature dual-axes scan head design with two collimated beams focused by a parabolic mirror. Scanning electron micrograph (SEM) of MEMS scanner is shown in Figure 2B. Figure 2C depicts the endomicroscope scanhead, including (a) two collimated beams focused by a parabolic mirror and (b) the endomicroscope with the 2D MEMS scanner. Figure 2D shows the endomicroscope passed through a 6-mm instrument channel of a special medical endoscope. This system was demonstrated both *ex vivo* and *in vivo* after topical application of indocyanine green, a near-infrared fluorescence dye. Real-time fluorescence imaging was performed in human colon, and Figure 2E shows a sequence of post-processed, mosaiced images

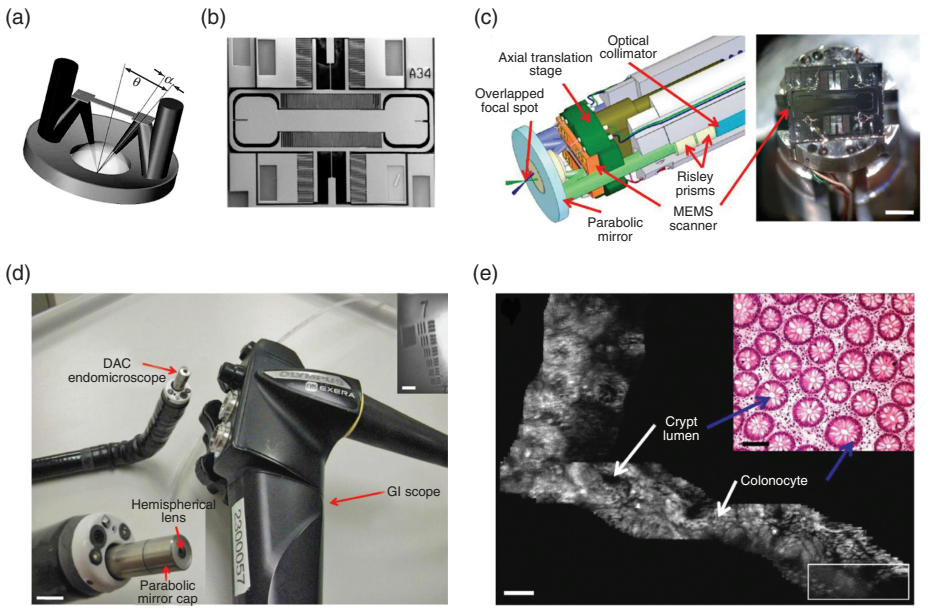


Figure 2. MEMS dual-axes confocal endomicroscope. (A) Miniature dual-axes scan head. (B) SEM of 2D MEMS scanner. (C) Endomicroscope scanhead. (D) Dual-axes confocal (DAC) endomicroscope delivered through an instrument channel of gastrointestinal (GI) endoscope. (E) A mosaic of normal colonic mucosa; inset shows histology (H&E). Scale bar = 100 μm. Used with permission [17, 18].

of normal colonic mucosa acquired at a depth of 60 µm and a representative histological (H&E) image of normal colonic mucosa.

2.2.2. Multi-photon Endomicroscope

Multi-photon-excited fluorescence imaging techniques have been developed to overcome limitations in tissue penetration depth experienced with single photon excitation. Multi-photon imaging systems use ultra-fast (femtosecond) laser pulses with near-infrared (NIR) excitation (typically 700 to 1040 nm), resulting in emission in the visible regime. As a result, multi-photon excitation systems offer key advantages that include intrinsic optical sectioning ability (owing to the non-linear excitation process and the restricted excitation volume), deeper tissue penetration depth, reduced out-of-focus photobleaching, and fewer associated phototoxicities. Multi-photon imaging has also attracted attention due to its ability to directly image tissue without the use of contrast agents. The intrinsic imaging contrast results from induced autofluorescence provided by endogenous fluorophores such as NADH, flavin and collagen. Besides providing high resolution imaging of tissue morphology, imaging based on endogenous fluorophores can provide clues about metabolic activity and matrix changes associated with diseases [19]. Safety is a key issue with endogenous fluorophores. Therefore, some factors such as endoscope design, laser intensity and tissue type should be considered carefully to avoid photobleaching, cell damage, and mutagenicity [20].

A number of different multi-photon endomicroscope designs have been developed [21–23] to investigate structure, function and molecular events [24, 25]. In one study, goblet cells, signifying the presence of intestinal metaplasia, were imaged using confocal and two-photon-excited fluorescence endomicroscopes [26]. Images collected with the two-photon endomicroscope exhibited higher resolution and contrast at each depth from the mucosal surface to 176 µm below in mouse intestinal specimens. In addition, the two-photon endomicroscope showed superior sectioning ability and less photobleaching. Some systems were not primarily designed for hollow organ imaging, but for biopsy guidance and/or replacement. In recent studies, an endoscope that uses a gradient index (GRIN) lens was developed for multi-photon and second harmonic generation (SHG) imaging, as shown in Figure 3A. The total system length was 26.9 cm with a rigid distal tip that is 1.2 mm in outer diameter, and ~8 cm in length (Figure 3B). This GRIN endoscope was validated *in vivo* in anesthetized rats [27]. An image of unstained superficial kidney renal cortex showed dark renal interstitium (RI), dark cellular nuclei (N), bright intrinsic fluorescent cytoplasm (CY), renal tubules (RT), renal capsule (RC), and dark blood filled lumen (L) inside the renal tubules (Figure 3C). This system has several advantages over flexible multi-photon endoscope. GRIN lens is inexpensive, small, and could be inserted into needles as small as 22 gauge. It also offered uniformed scan. In another recent study, multifocal multiphoton endoscope was developed to achieve fast frame rate without reducing signal-to-noise ratio per frame, and axial-sectioning [22]. The endoscope has a 3-mm-outer diameter and 4-cm-rigid length, and acquired images at 4 frames per second per focal plane with lateral and axial resolutions of 0.8 and 10 µm, respectively. The system was tested using excised mouse lung.

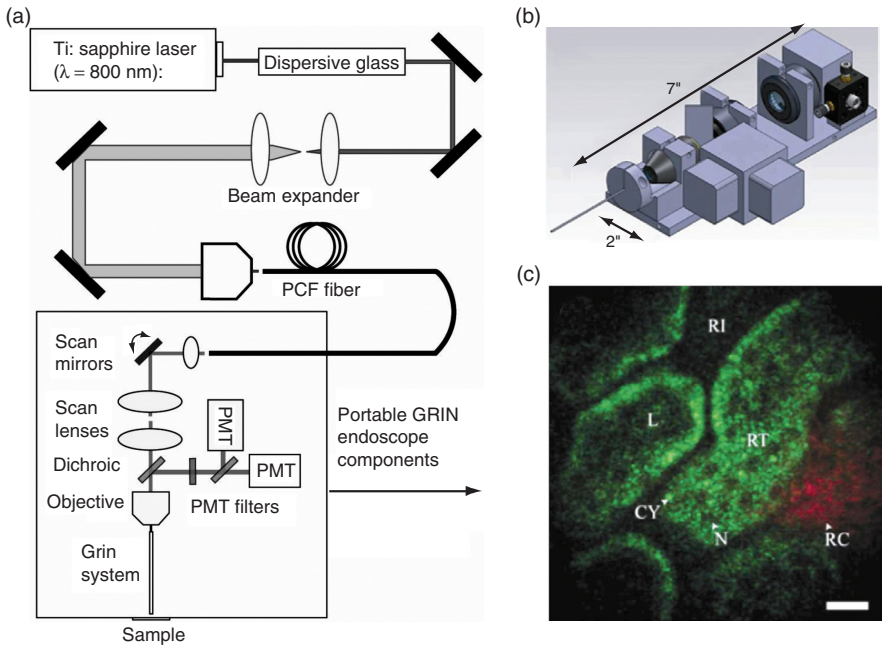


Figure 3. An optical diagram (A) and appearance (B) of portable GRIN endoscope. (PMT: photomultiplier tube, PCF: plastic-coated glass fibers.) (C) An unstained image of rat kidney renal cortex obtained using the GRIN endoscope; scale bar is 20 μM. Used with permission [27].

In addition, an integrated optical coherence tomography (OCT) and two-photon fluorescence endoscope was also developed for simultaneously acquiring tissue morphological and molecular information [28]. The system was tested using cell culture and excised mouse adipose tissue. In another study, a hybrid confocal and two-photon endomicroscope was developed, and demonstrated images of blood vessels labeled with rhodamine-B-dextran conjugates in a mouse ear [3]. Additionally, a high-NA endomicroscope was coupled to a multi-photon tomograph, *DermaInspect*. This integrated system has been used to detect skin cancer in a label-free manner [29], screen topically applied cosmetics and pharmaceuticals [30], and perform optical sectioning in pre-clinical studies [3].

2.2.3. Photoacoustic Endoscope

Recently, endoscopes have been developed based on the photoacoustic process [31], where energy from short laser pulses delivered into tissue is absorbed and converted into sound, resulting in deeper tissue penetration. Ultrasonic transducers collect the acoustic waves to form images. A hybrid optical/photoacoustic system has been demonstrated in a murine model using proflavine as a fluorescence contrast agent. An experimental setup for a hybrid photoacoustic and fluorescence endomicroscopic system is shown in Figure

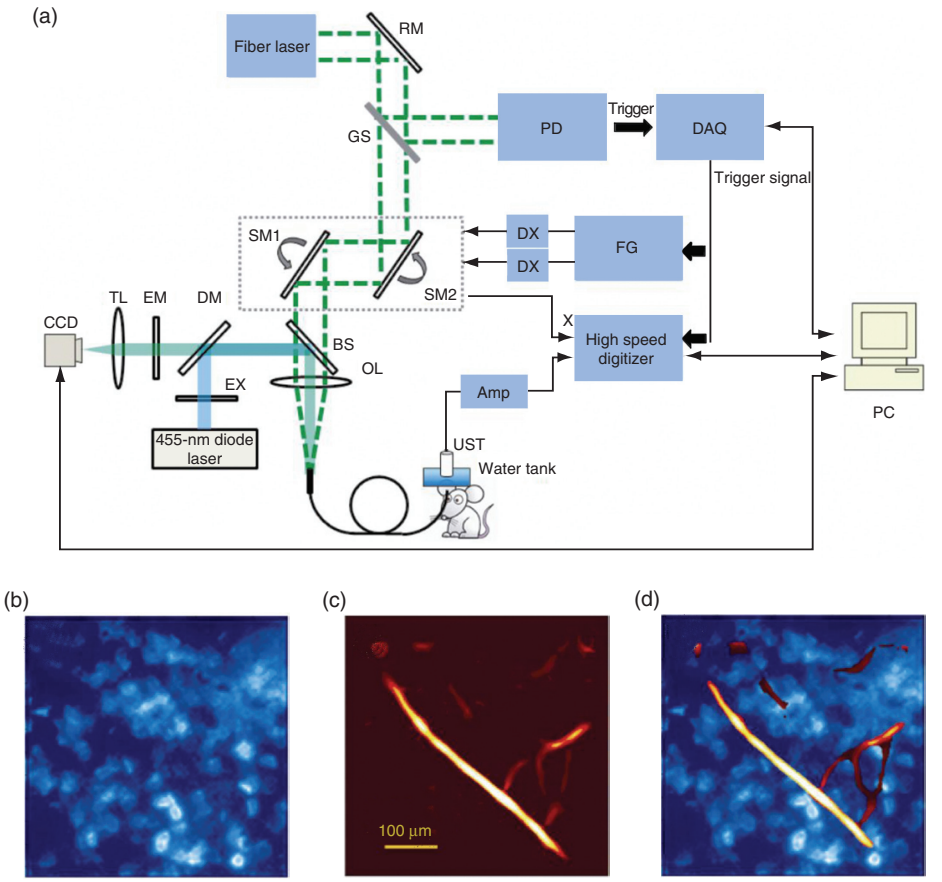


Figure 4. (A) Schematic diagram of the combined photoacoustic and fluorescence endomicroscopy imaging system. (RM: reflective mirror, GS: glass slide, PD: photodiode, DAQ: data acquisition, S/M 1 / 2: scanning mirrors 1 and 2, FG: function generator, DX/Y: galvanometer scanning mirror divers, BS: beam splitter, OL: objective lens, UST: ultrasound transducer, Amp: amplifier, CCD: charged-couple device, TL: tube lens, EM: emission filter, DM: dichroic mirror, EX: excitation filter.) (B) Fluorescence image of a mouse ear. (C) Optical-resolution photoacoustic endomicroscopy (OR-PAME) image of the same mouse ear. (D) Co-registered image of (B) and (C). Used with permission [31].

4A. The system collected fluorescence images to provide cellular morphology (Figure 4B) and photoacoustic (Figure 4C) images to visualize blood vessel structures. The two images were co-registered to provide multimodal information (Figure 4D). This technique may be used to monitor angiogenesis (the formation of new blood vessels) and effects of anti-cancer drugs on both cells and the microcirculation.

3. RECENT ADVANCES IN OPTICAL MOLECULAR PROBES

Probes have been developed to perform molecular imaging to improve the specificity of disease detection. These probes typically use a fluorophore attached to an affinity or biochemical ligand. Optical imaging probes can be categorized into three groups based on function: non-specific, targeted, and activatable. Advantages and disadvantages of different classes of molecular probes are presented in Figure 5.

Non-specific fluorophores are used without a targeting ligand. Examples of non-specific dyes include fluorescein, indocyanine green (ICG), and acriflavine. In general, these probes enhance contrast in mucosal morphology to identify pre-cancer and cancer. However, some dyes have a certain degree of intra- or extracellular localization; for example, acriflavine binds to nuclear materials. These fluorophores have low molecular weight (<1 kD), allowing for efficient delivery via topical or intravenous administration. The usefulness of imaging using of these contrast agents is limited by non-specific background.

Affinity ligands that bind specifically to cell surface targets are called 'targeted-cell-specific probes,' and include whole antibodies, antibody fragments, short sequence peptides, aptamers, and small molecules. Antibodies have been used extensively to detect tumors [32–35] using well-established fluorophore labeling methods. However, antibodies have slow binding onset (hours to days) due to steric hindrance from their

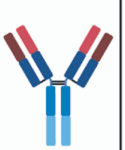
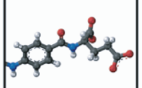
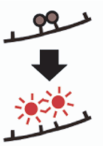
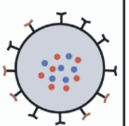
	Antibody	Peptide	Aptamer	Small molecule	Activatable probe	Nanoparticle
						
Advantages	- High specificity - Defined target - Approved therapeutic Ab may be labeled	- Low immunogenicity - Rapid tumor penetration - Rapid clearance - Low cost	- High specificity - Easy to produce and modify - Fast tumor penetration - Rapid clearance	- High specificity - Rapid clearance	- Specific activation - Optimized target - to-background ratios	- Loading with multiple targeting ligands - Loading with multiple therapeutics - Strong fluorescence
Disadvantages	- Reduced target-to-background ratios - Potential immunogenicity - High cost	- Variable affinity - Prone to degradation	- Undefined immunogenicity - High cost	- Fluorophore conjugation may alter pharmaco-kinetics and biodistribution	- Internalization frequently required for activation - Undefined safety profile	- Potential toxicity of non-biocompatible core - Renal clearance

Figure 5. Advantages and disadvantages of molecular probe platforms.

relatively large size (150–160 kDa) and typical geometry as well as from certain properties including conformation, surface charge, hydrophobicity, and hydrophilicity [36–38]. Another major limitation of antibodies as probes is slow clearance due to prolonged half-life resulting in reduced target-to-background ratios. In addition, most antibodies incur some immunogenicity with repeated use. Antibody fragments are smaller in size (~50 kDa), resulting in more efficient tissue penetration and accumulation in tumor cells and faster clearance, while retaining the specificity of the parent antibody. These properties offer improved target-to-background ratios and reduced immunogenicity.

Peptides can be developed with good specificity, high affinity for cellular targets, and rapid binding kinetics (few minutes). Because they contain only a few amino acids, peptides are less likely to be immunogenic. These properties are compatible with targeted imaging using endoscopy in a busy clinical unit. Also, peptides have shorter clearance times and much lower production costs compared to antibodies. Phage display is a powerful approach to screen for peptide sequences [31, 39]. This unbiased technique uses methods of recombinant DNA technology to generate a highly diverse library to find a specific binder to cell surface targets. This screening technique is also helpful for identifying new protein targets expressed by tumor cells. Natural peptides have a short biological half-life due to rapid degradation by peptidase and protease enzymes in plasma and tissues. Thus, once key amino acid residues have been identified, most peptides are structurally modified to prolong their *in vivo* half-life. Examples of modification methods include introduction of D-amino acids, use of unusual amino acids or side chains, integration of amino alcohol, and acetylation or amination of the N- and C- termini [40].

Aptamers are single stranded DNA, RNA, or modified nucleic acids that are generally developed through an *in vitro* selection process, such as SELEX (systematic evolution of ligands by exponential enrichment). Aptamers have low molecular weight (3–20 kDa), high specificity, fast tissue penetration, and rapid clearance. In addition, they are easy to discover, produce, and modify. However, they are expensive to produce in large quantities, and tailored modifications further increase production costs. A number of aptamer-based therapeutics are currently in clinical trials and have yet to elicit immunogenicity [41, 42].

Small molecules can also be used for binding to specific cell receptors. For example, folic acids bind to folate receptors, which are over-expressed on proliferating cells and macrophages. These probes have high specificity and rapid uptake. However, due to their small size, their pharmacokinetics and/or biodistribution may become affected after conjugation with fluorophores.

Activatable or "smart probes" do not emit light in their native form, and release fluorescence only in the presence of specific enzymes. Hence, they are known as 'enzyme-specific probes'. In general, these probes are less specific than targeted-cell-specific probes. They are frequently composed of multiple fluorophores attached on a polymer backbone. "Smart" probes have advantages that include a high target-to-background ratio, high loading capacity for fluorescent dyes, and ability to amplify fluorescence intensity upon multiple cleavage events. Smart probes can be activated by proteases, including

cathepsins and matrix metalloproteinases (MMPs), that have elevated levels in a number of tumors. These proteolytic enzymes facilitate tissue invasion, metastasis, and angiogenesis [43, 44]. The use of an NIR Cathepsin K (CatK)- activatable probe was demonstrated in a mouse model of atherosclerosis and resected human artheromata [45]. The probe consists of multiple CatK peptide substrates, GHPGGPQGKC, serving as linkers between NIR Cy5.5 dyes and a poly-L-lysine-PEG polymer backbone. The peptide substrate undergoes cleavage between the two glycine residues in the presence of CatK. In another study, an MMP-2- activatable probe was tested *in vitro* using photoacoustic microscopy [46]. The probe contains the MMP-2 peptide substrate, GGPLGMLARH, linked with a chlorophyll derivative or natural photosynthetic bacteriochlorophyll. MMP-2 cleaved the probe between the methionine and the glycine residues.

Several types of nanoparticles such as magnetic iron oxide, gold, quantum dots, and polymer nanoparticles have also been developed as non-specific contrast agents. Alternatively, these nanoparticles can also be used as molecule-specific probes by conjugating with targeting moieties, including peptides, antibodies, or aptamers. However, serious concerns about the potential toxicity of some nanoparticles such as quantum dots [47–50] have been raised. Therefore, nanoparticles should be thoroughly characterized in terms of toxicity, biodistribution, and pharmacokinetics before clinical use.

4. RECENT ADVANCES IN ENDOSCOPIC MOLECULAR IMAGING

By integrating with molecular probes, endoscopes and endomicroscopes may play an expanded role in the clinic. This section reviews recent findings that show potential applications of molecular imaging for disease detection in the clinical.

The two most common molecular probes that have been developed for clinical use are antibodies and peptides. Clinical use of antibodies as molecular probes has been widely explored. There are several known molecular targets for a variety of cancers, including epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), and human epidermal growth factor receptor 2 (HER2/neu). Specific antibodies to these molecular targets have been established and successfully applied in the clinic for targeted cancer therapy. Alternatively, these antibodies can be utilized as targeted imaging probes.

EGFR is a transmembrane glycoprotein tyrosine kinase receptor that promotes tumor proliferation, invasion, metastasis, and neovascularization [51]. EGFR is overexpressed in roughly 25–94% of colorectal cancer cases [52, 53] and in most squamous cell carcinomas of the head and neck [54]. Cetuximab and panitumumab are examples of monoclonal antibodies that bind specifically to EGFR. In one study, FITC-labeled EGFR antibodies were topically applied on excised human colonic specimens. The mean fluorescence of neoplasia was significantly higher than that of normal mucosa using confocal endomicroscopy [32].

VEGF is upregulated in tumor cells and function to promote angiogenesis. Bevacizumab is a well-known monoclonal antibody to VEGF that has been used to treat a number of solid tumors, including colorectal cancer [56] and head and neck tumors [57]. In one study, antibodies against VEGF and VEGFR-2 were labeled with Alexa Fluor 680. The imaging experiments were performed in colorectal mouse models (APC^{min} mice and xenografts), and in excised patient specimens using bioluminescence

imaging (IVIS) and confocal laser endomicroscopy [55]. Labeled antibody was injected to APC^{min} mice via tail vein and endoscopic imaging was performed 24 h post-injection. Examples of nuclear, cytoplasmatic and membrane staining on confocal are shown in Figure 6A-C, respectively. When stained with fluorescently labeled VEGF antibodies, staining is seen in the tumor cell cytoplasm but not in the nuclei (Figure 6B). Figure 6D shows a surgical specimen of a human liver colorectal cancer metastasis. Figure 6E shows a confocal image of VEGF identifying the margins of a liver metastasis. Only the tumor (asterisk *) was stained, whereas normal liver (cross +) revealed no signal. Confocal endomicroscopy using specific antibodies to VEGF showed a cytoplasmic distribution of VEGF that may be used for tumor detection. In another study, Bevacizumab-Cy5.5 conjugates were demonstrated in a mouse model of head and neck cancer. The labeled VEGF antibody was injected to tumor bearing mice via tail vein. Tumors were imaged 48 h post-injection and then resected. The result indicated tumor detection sensitivity of 80.9% and specificity of 91.7% [58].

Other antibodies used for head and neck cancer imaging include CD147 and transferrin receptor (TfR). CD147 is a membrane-spanning molecule that is highly expressed in head and neck tumor cells. Transferin receptor is a cell-membrane-internalizing receptor, which is responsible for iron sequestration and is overexpressed in many head and neck tumors. Anti-CD147 was conjugated with Cy5.5. The labeled anti-CD147 was injected into immunodeficient murine model bearing head and neck squamous cell carcinoma (HNSCC) via tail vein, and tumors were imaged using Leica stereomicroscope at 24, 48, 72, and 144 h after injection [59]. TfR antibody was labeled with Alexa-488. TfRNIR was injected into immunodeficient mice bearing HNSCC via tail vein, and animals were imaged using IVIS every 10–30 min up to at least 6 h [60]. The results indicated a potential use of anti-CD147 and TfR antibody for noninvasive head and neck tumor imaging.

Peptides play an important role as a class of molecular probes due to their safety, high specificity, and rapid binding kinetics. Alpha-v-beta-3 ($\alpha_v\beta_3$) integrin is an important adhesion molecule in the regulation of angiogenesis. This integrin can be found at the end of newly formed blood vessels and on many tumor cells. Arginine-glycine-aspartate (RGD) peptide is known to bind to $\alpha_v\beta_3$ integrin and has been used to target $\alpha_v\beta_3$ integrin. The cyclic RGD (cRGD) was conjugated to Cy5.5 or IRdye800CW for imaging of tumor angiogenesis [62]. In another study, a quenched cRGD molecule (RAFT-c(-RGDfK-)(4)-Cy5-SS-Q) was designed to be activated after being internalized [63].

The potential use of peptides has also been demonstrated in several pre-clinical and clinical studies. Using phage display, our group has developed several peptide sequences that are promising for molecular imaging and early detection of cancer. Recently, a fluorescently-labeled peptide QPIHPNNM was demonstrated *in vivo* using a wide-field small animal endoscope. The labeled QPI and control peptides were topically administered to polyps in distal rectal area. The distal rectal area was then washed and endoscopic imaging was performed after 5 min of incubation. The QPI peptide was shown to preferentially bind to dysplastic lesions in an Apc-mutation dependent (*CPC;Apc*) mouse model that spontaneously develops colonic adenomas,

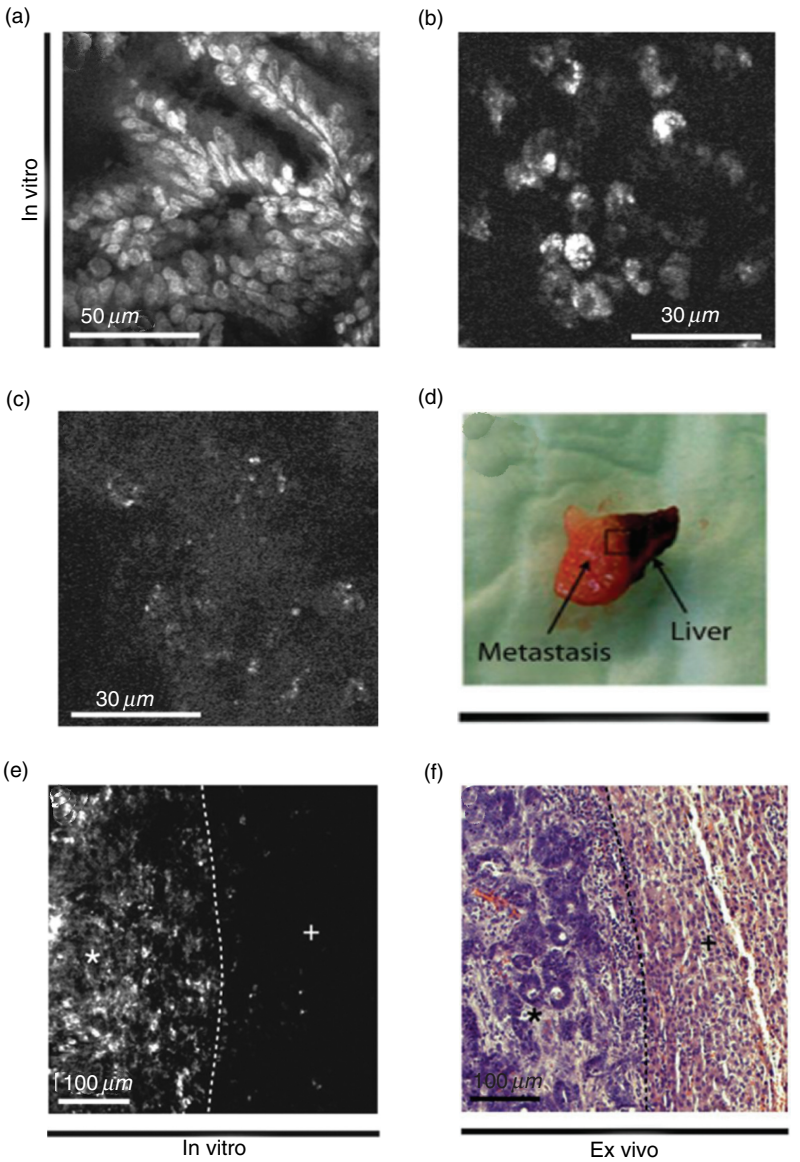


Figure 6. Confocal images of vascular endothelial growth factor (VEGF) and its receptor (VEGFR-2) in the same biopsy specimen of a human colorectal adenocarcinoma. (A) Non-specific nuclear and cellular staining using acriflavine. (B) VEGF-specific staining using labeled antibodies. (C) VEGFR-2-specific staining with labeled antibodies against the receptor. (D) Resection specimen of a human liver colorectal cancer metastasis. (E) Molecular imaging of VEGF identifies tumor margins. (F) Histology (H&E) of the same biopsy specimen. Used with permission [55].

but not to hyperplastic lesions in a mutant *Kras* mouse model [61]. The QPI peptide showed positive binding to multiple colonic adenomas (Figure 7A) and a single adenoma (Figure 7B) *in vivo*. The control peptide, GGGAGGGA, showed minimal binding (Figure 7C). In addition, the targeted peptide also showed minimal binding in a wild-type (control) mouse (Figure 7D) and the hyperplastic epithelium in a mutant *Kras* mouse model (Figure 7E).

In a recent study, multiple peptides labeled with different fluorescent dyes exhibited enhanced binding specificity and sensitivity in the *CPC;Apc* mouse model with a multispectral scanning fiber endoscope (SFE) [8] . The peptides KCCFPAQ, AKPGYLS, and LTTHYKL that bind specifically to colonic dysplasia were identified with *in vivo* phage display technique using *CPC;Apc* mouse model. The peptides were applied topically, endoscopic imaging was performed after 5 min of incubation, and specific binding of each peptide to colonic dysplasia was demonstrated (Figure 8A-C). The corresponding white light images are shown below (Figure 8D-F). The KCCFPAQ-DEAC and AKPGYLS-TAMRA peptides showed greater fluorescence intensity consistent with specific binding, compared to the control peptides GGGAGGG-DEAC and GGGAGGG-TAMRA with statistical significance. This study demonstrated the integration of a multispectral imaging system and multiple molecular probes that have potential to simultaneously visualize more than one gene target and differentiate the contribution of individual gene targets that are overexpressed in neoplasia. This potential may contribute to personalized imaged-guided therapy. Multispectral peptide probes can also be used to localize colonic dysplasia for early detection and improve margin detection for tumor resection.

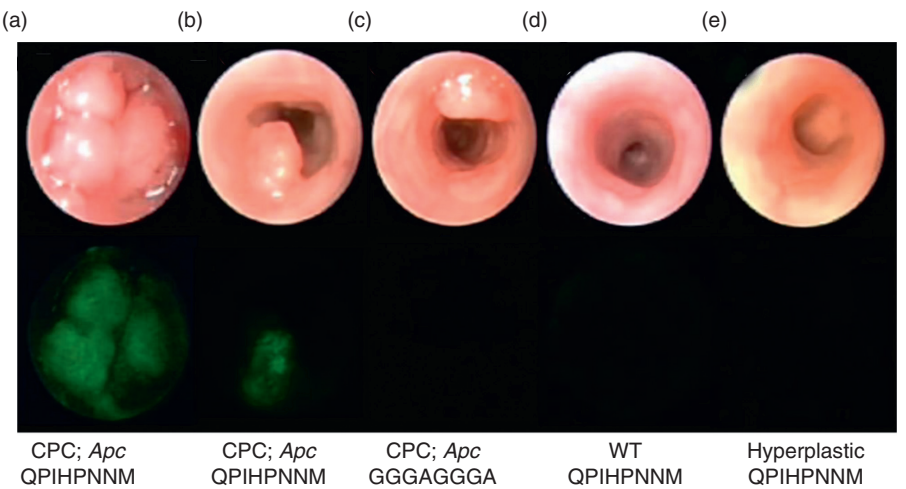


Figure 7. Images from wide-field endoscopy videos after application of fluorescence-labeled peptides. The top and bottom rows represent frames from white light and fluorescence, respectively. Used with permission [61].

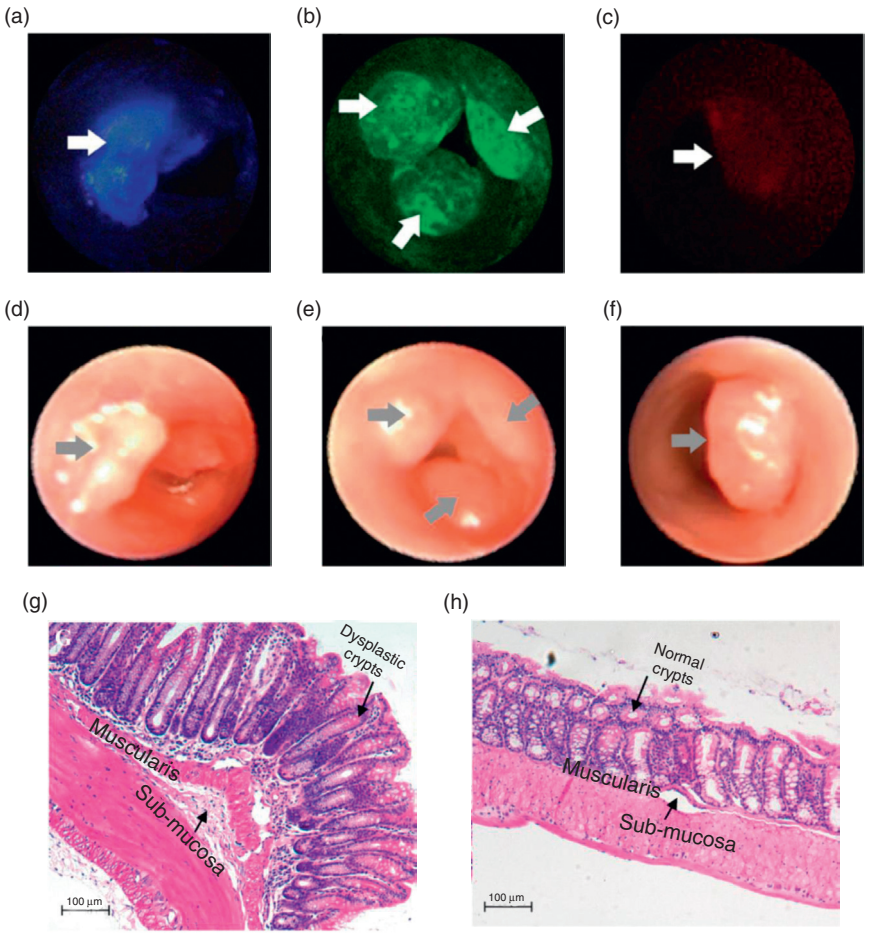


Figure 8. Wide-field fluorescence images of colonic adenoma collected with the multispectral scanning fiber endoscope after separate administration of peptides (A) KCCFPAQ-DEAC, (B) AKPGYLS-TAMRA, and (C) LTTHYKL-CF633. (D-F) The corresponding white light images. Histology (H&E) of (G) adenoma and (H) normal colonic mucosa. Used with permission [8].

5. DISCUSSION

A variety of novel endoscope and endomicroscope designs are being developed for real time imaging in pre-clinical and clinical applications. Conventional WLE provides a large FOV of the mucosal surface and are used as the standard of care for screening and surveillance. Advanced wide-field endoscopes such as AFI and NBI have been developed with enhanced spectral features. However, results for improved disease detection using AFI and NBI compared to WLE are variable, and depend on the study.

SFE is another wide-field endoscope design that has recently been developed for multispectral fluorescence imaging.

Confocal endomicroscopes have been employed to achieve imaging of sub-cellular features *in vivo* in numerous clinical studies. Currently, the single axis designs are limited by short WD, small FOV, and superficial tissue penetration depths with 488 nm excitation. Recently, there have been significant efforts to develop endomicroscopes that have improved tissue penetration depths without compromising in resolution, such as the dual-axes confocal and multiphoton endomicroscopes. These instruments are capable of performing optical sectioning in real time, which potentially allows for disease detection at the point of care. Two-photon fluorescence endomicroscopy is also a powerful technology for imaging of unstained biological tissues. Besides providing high-resolution, *in vivo* images, this label free instrument has shown great medical diagnostic promise. However, the maximum imaging depth is limited to ~1 mm [27].

A number of molecular probes have been developed to improve disease detection capability and enhance the clinical utility of existing endoscopes. Targeting agents are designed to achieve high specificity, strong signal, pharmacokinetic profile compatible with clinical use, and high target-to-background ratios. In addition, safety is a key requirement for translating these exogenous agents for human use. The process of developing imaging agents is analogous to drug development process that is very costly, high risk, and time consuming. It takes about 10 to 17 years and nearly $0.8–$1.7 billion to bring a therapeutic drug to market [64], while the current estimate of cost to bring a new imaging agent to the market is about $100 to $500 million [65]. To date, there are only a limited number of fluorescence dyes, such as fluorescein isothiocyanate (FITC) and indocyanine green (ICG), approved by FDA. In addition, 5-aminolevulinic acid (5-ALA) was approved in the European Union for cystoscopy [66].

Currently, novel endoscopic imaging methods are being developed taking advantage of the rapid technological advances in micro-optics, scanning and actuation mechanisms, light sources, and emerging molecular probes. Future endoscopic instruments are likely to include multiple modalities such as 1) high-resolution white-light imaging for rapid wide-field observation, 2) high-contrast fluorescence imaging for highlighting disease regions using molecular probes, and 3) microscopic imaging to validate receptor status at a sub-cellular level. These future endoscopes promise to allow physicians to make more accurate clinical decisions, reduce time for diagnosis, guide tissue sampling, and ultimately improve patient outcomes.

6. CONCLUSION

Endoscopes are important tools for imaging the mucosa of hollow organs *in vivo* for screening and surveillance of several common cancers in organs such as esophagus, stomach, colon, and rectum. Rapid technological advances in light sources, micro-optics, optical fibers, and miniature scanners will further expand these capabilities through multi-spectral image collection, greater access to internal organs, better tissue penetration, and 3D volumetric imaging. The addition of molecular probes provides a new feature that allows clinicians to visualize pre-cancerous and cancerous lesions based on their expressed protein targets rather than on morphology alone. These

innovative target-specific molecular probes promise to significantly improve the specificity of disease detection, and may assist physicians to detect cancer at an earlier time point before gross anatomical changes occur. Other potential benefits of these developments include imaged guide therapy, targeted therapy and personalized medicine.

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CONFLICT OF INTEREST

The authors have no conflicts or financial relations to disclose.

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